






MicroRNA-17-5p Reduces Inflammation and Bone Erosions in Mice With Collagen-Induced Arthritis and Directly Targets the JAK/STAT Pathway in Rheumatoid Arthritis Fibroblast-like Synoviocytes

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Objective. To examine microRNA (miRNA) expression across rheumatoid arthritis (RA) phenotypes, along with the effects and mechanisms of action of miRNA-17-5p (miR-17).

Methods. A miRNA array was performed in synovial tissue biopsied from patients with treatment-naïve erosive RA (n = 3) and those with treatment-naïve nonerosive RA (n = 3). MicroRNA-17 lipoplex was delivered intraarticularly in the murine collagen-induced arthritis model. Clinical, histologic, and structural effects were studied over the course of arthritis. In-depth studies of the mechanisms of action of miR-17 were performed in primary RA fibroblast-like synoviocytes (FLS) isolated from synovial tissue.

Results. Fifty-five miRNAs including miR-17 were reduced in erosive RA. The miR-17 transfection into arthritic paws reduced the clinical inflammation score between day 2 and day 7 (1.9 versus 2.8; $P = 0.03$). Synovial B cell, T cell, macrophage, and polynuclear neutrophil infiltration was significantly reduced. Structural damage was also decreased, as shown by a reduction in the number of osteoclasts detected using tartrate-resistant acid phosphatase staining (osteoclast surface/bone surface 18% versus 32%; $P = 0.005$) and erosion score by computed tomography analysis (1.7 versus 2.9; $P = 0.023$). Proinflammatory cytokines from the interleukin-6 (IL-6) family and IL-1 β expression were also significantly reduced, but tumor necrosis factor was not. MicroRNA-17 directly targeted the 3'-untranslated regions of STAT3 and JAK1. STAT3 and JAK1 messenger RNA (mRNA) and protein expression were reduced in RA FLS following miR-17 transfection. STAT3 and JAK1 mRNA and activation of STAT3, as assessed by immunohistochemistry, were also reduced in injected paws (% stained area 0.62% versus 0.93%; $P = 0.035$).

Conclusion. We demonstrate an antiinflammatory and antierosive role of miR-17 in vivo. This effect involves the suppression of the IL-6 family autocrine-amplifying loop through the direct targeting of JAK1 and STAT3.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease of the joints, affecting 0.5–1% of the population worldwide (1). Inflammatory cytokines including tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-17, and IL-6 are part of the inflammatory milieu

in RA (2). Most notably, they are potent stimulators of synovial fibroblasts, cells that are important contributors to and regulators of inflammation through their production of inflammatory cytokines such as IL-6 family cytokines (IL-6, leukemia inhibitory factor [LIF]) and chemokines (3). Synergistic effects and autocrine amplification loops involving the IL-6/JAK/STAT pathway have been

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observed with these cytokines in fibroblasts, resulting in chronic inflammation (3,4). Although biologic drugs (e.g., anti-TNF, anti-IL-6 receptor [IL-6R], anti-IL-17) and small molecules (JAK inhibitors) have transformed patient care, reducing both inflammation and joint destruction, 30% of patients switch or discontinue their biologics in the first year of treatment due to nonresponse (5). These data highlight the need for innovative therapies, especially for patients with aggressive and erosive RA (6).

A field of research currently in full expansion is the study of microRNA (miRNA or miR) in chronic inflammatory rheumatic diseases, particularly RA (7). Alterations in miRNA expression are involved not only in tumor development but in inflammatory processes such as rheumatic diseases. The miR-17-92 cluster has been the subject of intense research in recent years due to its key role in cancer development and progression. The MIR17HG gene (miR-17-92 cluster host gene [non-protein coding] located on chromosome 13 [13q31.3]) encodes a polycistronic primary transcript that yields ≥ 6 mature miRNAs: miR-17, miR-18a, miR-19a, miR-19b, miR-20, and miR-92 (8). Beyond its involvement in oncology (9), this cluster has more recently been studied in rheumatic diseases. Expression of miR-17-5p is reduced in synovial fibroblasts, peripheral blood mononuclear cell (PBMCs), and synovial tissue from RA patients, suggesting a crucial role in RA pathogenesis (10). In vitro, the transfection of pre-miR-17 into RA fibroblast-like synoviocytes (FLS) has an antiinflammatory effect through direct targeting of the MAP3K5 kinase (apoptosis signal-regulating kinase 1 [ASK1]) in the TNF signaling pathway, thus reducing IL-6 production (10).

To date, however, the mechanisms involved in regulating the antiinflammatory effect of miR-17 remain unclear. Moreover, no studies have investigated the therapeutic potential and mechanism of action of the miR-17-92 cluster in murine models of arthritis. We undertook this study to assess the expression of the miR-17-92 cluster in different RA phenotypes (erosive and nonerosive), to further elucidate the mechanisms and direct targets involved in the antiinflammatory role of miR-17-5p, and to investigate the therapeutic effects of miR-17-5p in arthritis.

MATERIALS AND METHODS

Patient recruitment, synovial membrane miRNA array, and RNA sequencing of arthritic paws are described in detail in Supplementary Methods (available on the *Arthritis & Rheumatology* website at <http://onlinelibrary.wiley.com/doi/10.1002/art.41441/abstract>).

The RNA sequencing data discussed here have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession no. GSE139269 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE139269>).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Human and mouse tissues were homogenized using a D125 Ultra-Turrax homogenizer (IKA) in QIAzol lysis reagent (Qiagen). Cell cultures were lysed by scraping

in QIAzol. Total RNA, including miRNA, was isolated using a miRNeasy Kit (Qiagen). For messenger RNA (mRNA) and miRNA, RT-qPCR was carried out as previously described (11,12). Analysis was performed using hypoxanthine guanine phosphoribosyltransferase (for mRNA) or RNU6B (for miRNA) as invariant control, and results were expressed as $2^{-\Delta C_t}$.

Collagen-induced arthritis (CIA) mouse model. Animal experimentation was performed according to institutional guidelines and was approved by the French ethical committee CEEA.PdL.06 and by local veterinary services. Eight-week-old male DBA/1 mice were immunized as previously described (11). Lipoplexes of miR-17-5p mimic or irrelevant miRNA control (miRvana; Thermo Fisher Scientific) were prepared using Lipofectamine (RNAiMAX Transfection Reagent) according to the instructions of the manufacturer (Thermo Fisher Scientific). Intraarticular injections of miRNA lipoplex (8×10^{-12} moles of miRNA per injection) were administered into arthritic ankles with 30-gauge needles at the first signs of inflammation (day 1) and then every 48 hours. In each animal, only hind paws were considered for injection, and ankles were considered independently. Animals with arthritis were randomized into different groups (noninjected mice with arthritis, miRNA control-injected mice with arthritis, anti-miRNA control-injected mice with arthritis, miR-17-injected mice with arthritis, and anti-miR-17-injected mice with arthritis). The anti-miR-17 and miR-17 experiments were conducted as independent experiments.

The injection protocol was defined through prior experiments in order to select the best concentration, volume, and frequency of injections. Inflammatory symptoms were assessed daily by clinical scoring, and mice were euthanized on day 4 or day 7 (peak phase). Structural damage (chondrolysis, bone erosions) was analyzed with a high-resolution SkyScan 1076 micro-computed tomography (micro-CT) system (11). Semiquantitative scores (0–4) were obtained for chondrolysis and bone erosions after 3-dimensional reconstruction. Ankles were then fixed, decalcified, and embedded. All histologic staining was performed as previously described (11). For clinical, histologic, and micro-CT analysis, researchers were blinded with regard to treatment group.

Cell culture. Primary RA FLS were isolated from synovial membranes obtained from consenting RA patients undergoing arthroplasty (11). Primary RA macrophages were generated from RA synovial fluid monocytes using granulocyte-macrophage colony-stimulating factor/interferon- γ (IFN γ), as previously described (13). THP-1 monocytic cells (American Type Culture Collection) were stimulated with phorbol 12-myristate 13-acetate (Sigma) to induce differentiation into active macrophage-like cells, as described (11). MicroRNA-17-5p mimic or irrelevant miRNA control was transfected for 24 hours at a final concentration of 6 nM using Lipofectamine, according to the instructions of the manufacturer. Cells were stimulated with IL-1 β (1 ng/ml; R&D systems), TNF (10 ng/ml; R&D systems), or

lipopolysaccharide (LPS) (10 or 100 ng/ml; Sigma) for 24 hours and then lysed in radioimmunoprecipitation assay buffer. Primary antibodies (all from Cell Signaling Technology) were used to detect proteins of interest by Western blotting, as previously described (11). Enzyme-linked immunosorbent assay for the detection of IL-6 in culture supernatants was performed according to the instructions of the manufacturer (R&D Systems).

Luciferase reporter assays. Luciferase assays were performed as previously described (14,15) and are described in Supplementary Methods (<http://onlinelibrary.wiley.com/doi/10.1002/art.41441/abstract>).

Statistical analysis. To assess the significance of differences between groups, Mann-Whitney U tests or paired Wilcoxon-Mann-Whitney tests were performed using GraphPad Prism, version 6. *P* values less than 0.05 were considered significant. Data are presented as the mean ± SEM, and 95% confidence intervals (95% CIs) were calculated.

RESULTS

Differential expression of miRNAs depending on RA phenotype. We assessed the expression of 754 known miRNAs by performing low-density TaqMan arrays in knee synovial

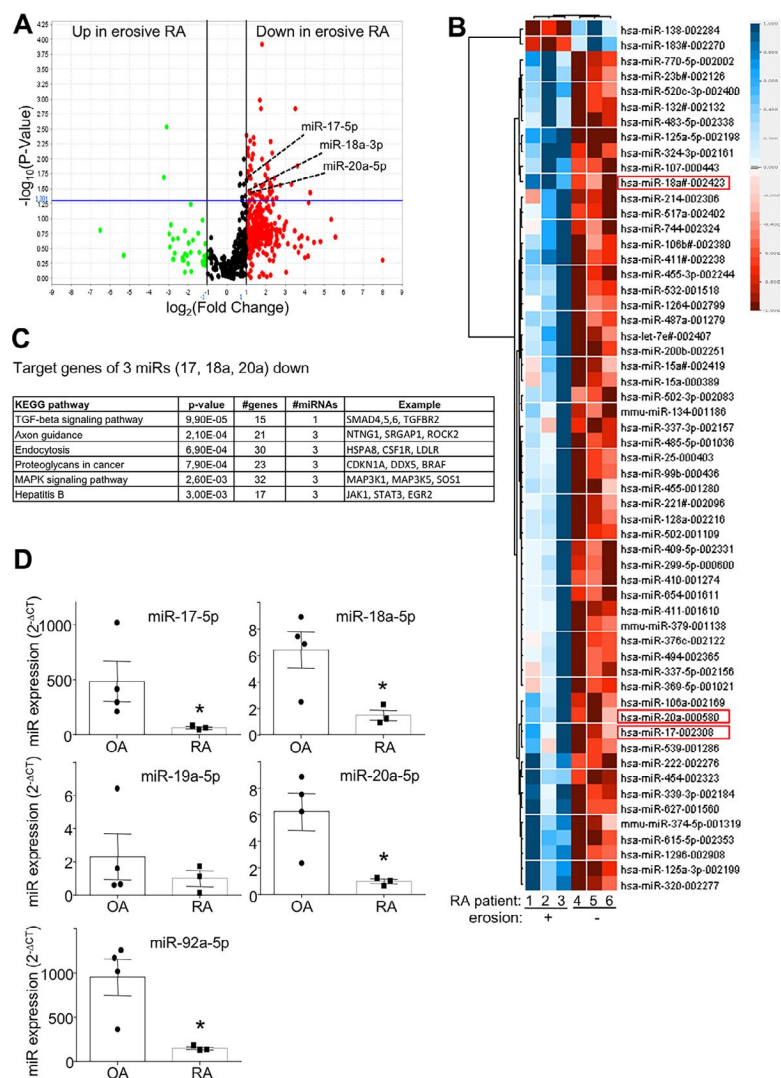


Figure 1. Expression of the microRNA-17-92 (miR-17-92) cluster is decreased in erosive rheumatoid arthritis (RA). **A**, Volcano plot showing differential expression of 754 miRNAs in erosive RA synovium (n = 3) and nonerosive RA synovium (n = 3), as assessed by TaqMan array. Up-regulated miRNAs (>2-fold change) in erosive RA are shown in green, down-regulated miRNAs in red. Three miRNAs from the miR-17-92 cluster were significantly down-regulated in erosive RA (*P* < 0.05). **B**, Hierarchical clustering of ΔC_t values of the top 51 differentially expressed miRNAs (>2-fold change; *P* < 0.05) in erosive and nonerosive RA synovium. The 3 miRNAs from the miR-17-92 cluster are shown with red borders. **C**, Target genes and pathways of miR-17, miR-18a, and miR-20a as analyzed with DIANA-mirPath version 3 software. **D**, Down-regulated expression of the miR-17-92 cluster in RA synovium (n = 3) compared to osteoarthritis (OA) synovium (n = 4), as assessed by reverse transcription-quantitative polymerase chain reaction. Symbols represent individual subjects; bars show the mean ± SEM. * = *P* < 0.05 by Mann-Whitney U test. TGF = transforming growth factor.

samples from patients with erosive RA ($n = 3$) (≥ 1 erosion visible on plain hand and feet radiographs) and from patients with nonerosive RA ($n = 3$). All patients had early RA, were naive to treatment with disease-modifying antirheumatic and biologic drugs, and were seropositive for both rheumatoid factor and anti-citrullinated protein antibody (Supplementary Table 1A, <http://onlinelibrary.wiley.com/doi/10.1002/art.41441/abstract>). The median disease duration was 2.3 months.

Fifty-five miRNAs were significantly down-regulated in erosive RA and only 2 miRNAs were up-regulated (>2 -fold change) (Figures 1A and B). These dysregulated miRNAs are known to target numerous genes implicated in various pathways apparently unrelated to bone erosion, such as thyroid hormones or proteoglycans in cancer (Supplementary Table 2, <http://onlinelibrary.wiley.com/doi/10.1002/art.41441/abstract>). Interestingly, 3 miRNAs from the miR-17-92 cluster were significantly down-regulated in erosive RA: miR-17-5p (2.15-fold change [95% CI 1.3–3.5]), miR-18a-3p (2.2-fold change [95% CI 1.3–3.56]), and miR-20a-5p (2.4-fold change [95% CI 1.3–4.2]). These 3 miRNAs are known to target genes implicated in transforming growth factor β (*SMAD4*, *SMAD5*, *SMAD6*, *TGFBR2*) or MAPK (*MAP3K1*, *MAP3K5*) signaling pathways or in hepatitis B (*JAK1*, *STAT3*) (Figure 1C), i.e., genes that have previously been described in inflammatory or bone remodeling processes.

In order to assess the expression of miRNAs from cluster 17-92 across disease states, RT-qPCR was performed in synovial tissue from a different cohort of patients with either nonerosive osteoarthritis (OA) ($n = 4$) or established erosive RA ($n = 3$) (mean disease duration 6 years) (Supplementary Table 1B, <http://onlinelibrary.wiley.com/doi/10.1002/art.41441/abstract>). Once again, miR-17-5p, miR-18a-5p, and miR-20a-5p, as well as miR-92a-5p, were down-regulated in erosive RA synovium compared to OA synovium (Figure 1D). Interestingly, findings from prior studies in RA FLS (10) have suggested an antiinflammatory role of miR-17-5p (hereafter referred to as miR-17), and we sought to study examine the role and mechanisms of action of miR-17 in more detail.

Antiinflammatory role of miR-17 in the CIA mouse model. Arthritic paws (score ≥ 1) of mice with CIA were injected intraarticularly with either irrelevant miRNA (miRNA control) or miR-17 lipoplex every other day. At the peak of inflammation (day 7, after 3 miRNA injections), paws injected with miR-17 lipoplex displayed an increase of miR-17 expression compared to control-injected paws (Figure 2A), whereas endogenous miR-17 expression was significantly reduced in arthritic ankles compared to nonarthritic ankles. The injection of miR-17 significantly reduced inflammation scores beginning on day 2, compared to the miRNA control group, and this effect persisted through day 7 (Figure 2B). There was no significant difference in arthritis scores between the group injected with miRNA control and the noninjected group.

Injection of miR-17 also significantly reduced the synovial inflammation score (obtained by hematoxylin and eosin staining) and specific synovial cell infiltration including CD45R+ B cells, CD3+ T cells, Iba1+ macrophages, and Ly6G+ polynuclear neutrophils (Figures 2C and D). No increase of cell apoptosis was observed in the miR-17-injected group (Supplementary Figure 1A, <http://onlinelibrary.wiley.com/doi/10.1002/art.41441/abstract>). Injection of miR-17 significantly reduced the joint erosion score (assessed by micro-CT), cartilage degradation, and osteoclast surface/bone surface percentage (assessed by tartrate-resistant acid phosphatase staining) (Figures 2C and E). We also performed injections of miR-17 lipoplex in mice with CIA that were euthanized at the resolution phase of arthritis (day 14) instead of during the peak phase. We observed a significant reduction in clinical scores, mostly at the peak phase of inflammation (days 2–10) (Supplementary Figure 1B). Notably, when mice were injected with anti-miR-17 in order to neutralize the endogenous miR-17 in arthritic paws, we observed a significant increase in clinical arthritis scores from day 3 to day 11 (Supplementary Figure 1C).

MicroRNA-17 acts by targeting the IL-6 pathway. In order to gain insight into the mechanism of action of miR-17, we assessed gene expression through RNA sequencing in miR-17-injected paws at the peak of inflammation (Figures 3A and B). We found 1,289 genes that were differentially expressed in miR-17-injected paws: 546 up-regulated genes and 743 down-regulated genes. Interestingly, enrichment of IFN-responsive genes was observed for genes up-regulated by miR-17 (*Ifit3*, *Ifit3b*, *Irf7*, *Stat1*), as well as genes implicated in antigen processing (*B2m*, *H2-K1*) or in ossification (*Col2a1*, *Mgp*) (Figures 3B–D and Supplementary Table 3, <http://onlinelibrary.wiley.com/doi/10.1002/art.41441/abstract>). Moreover, enrichment of genes related to inflammatory response (*Cd14*, *Nlrp3*), osteoclast differentiation (*Trap*, *Nfatc1*, and the matrix-remodeling enzymes *Ctsk* and *MMP9*), neutrophil chemotaxis (*Cxcl1*, *Cxcr2*), or cytokine-receptor interaction was observed among down-regulated genes. Of interest, several genes from the IL-6 family and/or STAT3 target genes were down-regulated by miR-17 (*Il6*, *Lif*, *Osm*, *Ccl2*, *Ccl20*, *Socs3*) (Figures 3B, C, and E and Supplementary Tables 3 and 4, <http://onlinelibrary.wiley.com/doi/10.1002/art.41441/abstract>).

We next confirmed by RT-qPCR that the expression of mRNA for genes encoding IL-6 family cytokines and/or STAT3 target genes (*Il6*, *Osm*, *Rankl*) was significantly reduced in ankles injected with miR-17 lipoplex (Figure 3E). IL-1 β transcripts were also significantly reduced, but TNF was not (Figure 3F).

Reduction of IL-6 (but not of macrophage) secretion in RA FLS by miR-17. It appears that miR-17 is prominently expressed in endothelial cells. Its expression in fibroblasts appears to be variable, while lower expression levels were observed in neutrophils, T cells, and B cells (Supplementary Figure 2, <http://onlinelibrary.wiley.com/doi/10.1002/art.41441/>

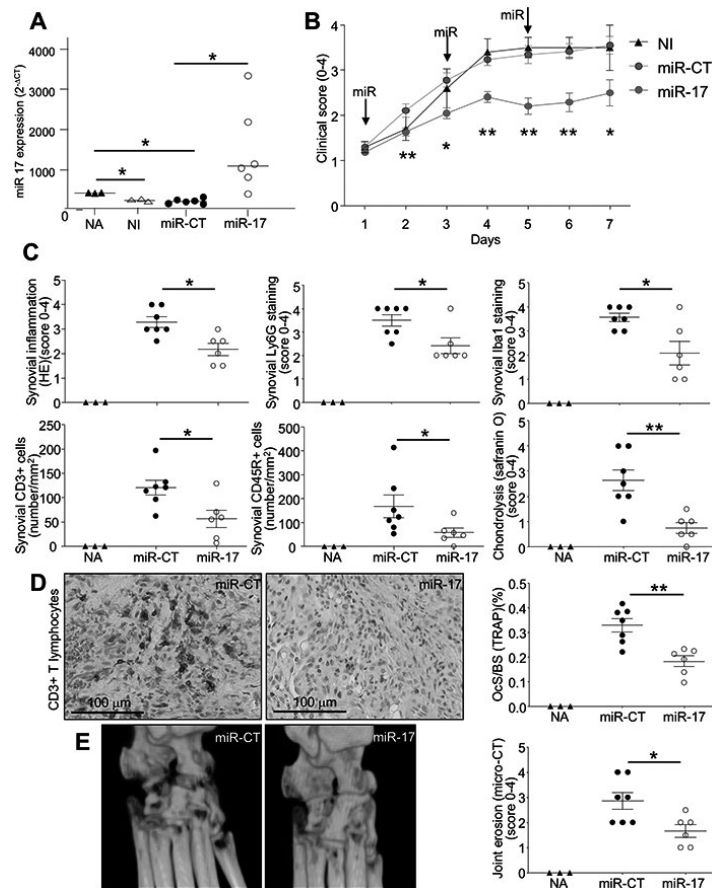


Figure 2. Effect of microRNA-17 mimic lipoplex (miR-17) injection into the ankles of mice with collagen-induced arthritis (CIA). **A**, On day 7, miR-17 was overexpressed in the ankles of mice with CIA that were injected with the miR-17 lipoplex. **B**, A significant reduction in clinical arthritis scores in miR-17-injected ankles is shown. **C**, On day 7, miR-17 significantly reduced synovial inflammatory infiltrate and joint erosion, as shown by inflammation scores obtained using hematoxylin and eosin (H&E) staining, as well as Ly6G and Iba1 staining scores. Synovial CD45R+ and CD3+ positive cells are shown. Osteoclast surface/bone surface (OcS/BS) percentage was determined with tartrate-resistant acid phosphatase (TRAP) staining, using NDPView2 software. Chondrolysis was evaluated based on Safranin O staining scores. Joint erosions were analyzed by micro-computed tomography (micro-CT) using Skyscan CtVol software. Scores for the talocrural joint from the micro-CT analysis were used. Symbols represent individual ankles; bars show the mean \pm SEM. * = $P < 0.05$; ** = $P < 0.005$, by Mann-Whitney U test. **D**, Representative immunohistochemistry images of CD3+ T lymphocytes are shown. **E**, Representative micro-CT images of joint erosions are shown. No damage from the injection was observed clinically, histologically, or structurally. NA = nonarthritic, noninjected ankles ($n = 3$); NI = arthritic noninjected ankles ($n = 3$); miR-CT = arthritic ankles injected with irrelevant miRNA lipoplex control ($n = 6$ or $n = 7$); miR-17 = arthritic ankles injected with miR-17 ($n = 6$). Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.41441/abstract>.

abstract). Moreover, miR-17 was previously shown to repress IL-6 production in RA FLS in culture. RA FLS were efficiently transfected with miRNA lipoplex (>95% of transfected cells) and transfection of miR-17 led to 100–1,000-fold increased expression of miR-17 (Supplementary Figures 3A and B, <http://onlinelibrary.wiley.com/doi/10.1002/art.41441/abstract>). Overexpression of miR-17 did not alter RA FLS survival or proliferation (Supplementary Figure 3C). After 24 hours of stimulation with IL-1 β , TNF, or LPS, we confirmed that overexpression of miR-17 significantly reduced IL-6 secretion in RA FLS from 4 different patients (Figure 4A). However, transfection of anti-miR-17 showed overall no effect on IL-6 secretion (data not shown). A low level of endogenous miR-17 expression in RA FLS may have precluded its efficient neutralization with anti-miR-17 (10).

In order to determine whether miR-17 could have an effect in other immune cells, we studied the effect of miR-17 transfection in RA macrophages generated from RA synovial fluid monocytes. Although RA or THP-1 macrophages were efficiently transfected by miRNA lipoplex (>90% of transfected cells) and by miR-146 (used as positive control) (14), the LPS-induced secretion of IL-6 was not affected by miR-17 transfection (Figure 4B), in contrast to the findings in RA FLS (Figure 4A).

Reduced PBMC migration and osteoclastogenesis in miR-17-transfected RA FLS. We studied the role of miR-17 in RA FLS on immune cell migration and osteoclastogenesis. PBMC migration was reduced significantly in wells containing miR-17-transfected RA FLS (Figure 4C). After 14 days, the number of

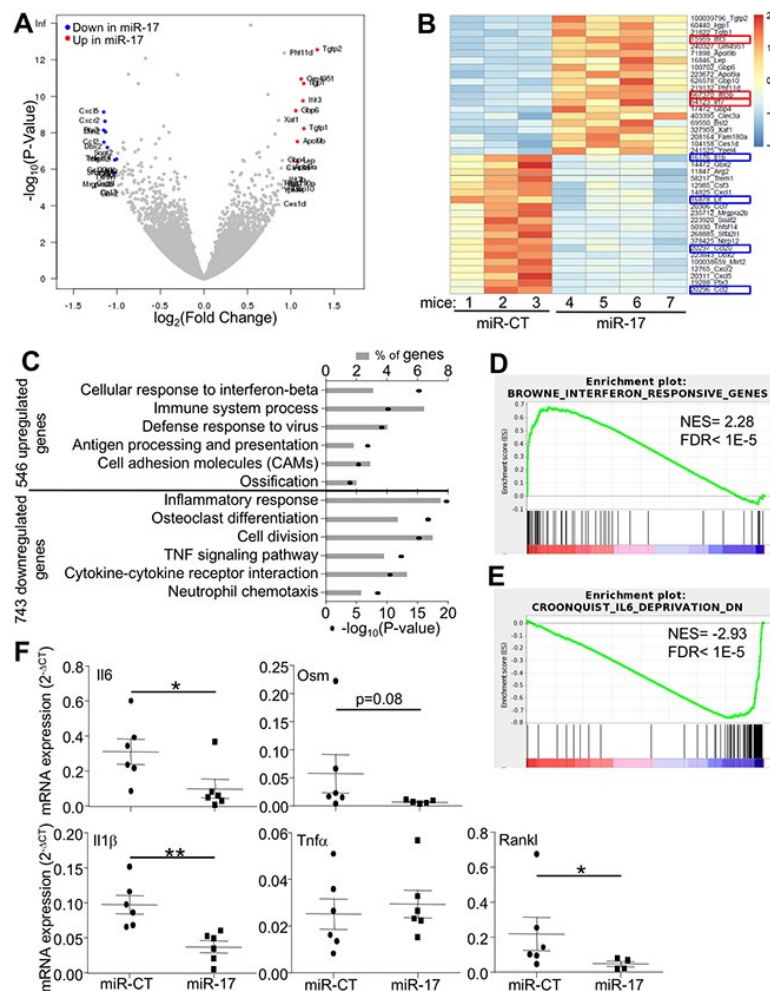


Figure 3. MicroRNA-17 suppresses expression of interleukin-6 (IL-6) family cytokines and STAT3 target genes in the ankles of mice with CIA. **A**, Volcano plot showing differential expression of genes in the ankles of mice with CIA, as assessed by RNA sequencing on day 7 after miR-17 lipoplex injection. The top 8 up-regulated genes (>2-fold change; adjusted $P < 0.01$) in ankles injected with the miR-17 mimic lipoplex ($n = 4$) compared to ankles injected with irrelevant miRNA control ($n = 3$) and the top 10 down-regulated genes are shown. Differential expression analysis was performed with DESeq2. **B**, Heatmap representation of the top 40 differentially expressed genes (adjusted $P < 0.01$). **C**, Enriched biologic processes and pathways, determined using DAVID ontology software on all up-regulated and down-regulated genes (adjusted $P < 0.01$). **D** and **E**, Gene Set Enrichment Analysis (GSEA) performed on all differentially expressed genes (1,289 genes; adjusted $P < 0.01$). Enrichment of interferon-responsive genes for genes up-regulated by miR-17 (**D**) and enrichment of genes related to IL-6 deprivation for those down-regulated by miR-17 (**E**) are highlighted. **F**, Confirmation of down-regulated expression of genes encoding IL-6 family cytokines and/or STAT3 target genes in ankles injected with miR-17 lipoplex ($n = 6$ per group), as assessed by reverse transcription-quantitative polymerase chain reaction. Symbols represent individual ankles; bars show the mean \pm SEM. * = $P < 0.05$; ** = $P < 0.005$, by Mann-Whitney U test. TNF = tumor necrosis factor; NES = normalized enrichment score; FDR = false discovery rate (see Figure 2 for other definitions). Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.41441/abstract>.

osteoclasts was significantly reduced in wells supplemented with conditioned media from RA FLS transfected with miR-17 compared to miRNA controls (Figure 4D). These findings suggest that transfection with miR-17 in RA FLS could be responsible for the inhibition of immune cell migration in the synovial tissue, along with reduction in erosions, via suppression of osteoclastogenesis.

JAK1/STAT3 pathway directly targeted by miR-17. In

order to gain further insight into the miR-17 mechanism of action, we analyzed the expression of genes that could be implicated in IL-6 production in RA FLS that were stimulated with TNF. IL-6 and

LIF transcripts were significantly reduced in miR-17-transfected RA FLS (Figure 5A). The other IL-6 family members, oncostatin M (OSM) and IL-11 or the osteoclastogenic cytokine RANKL, which were also found to be down-regulated by miR-17 in arthritic paws, were not detected in RA FLS. No difference in expression of the receptor chain's LIF receptor (LIFR), OSM receptor, or gp130 was observed. To further depict miR-17 direct targets, we performed in silico analyses (TargetScan and DianaLab). Most of the genes described as potential miR-17 targets belonged to the TNF and IL-6 family signaling pathways such as *ASK1*, *TRAF2* (10), *JAK1* (15), *STAT3* (16), or several SMAD pathways (17), among

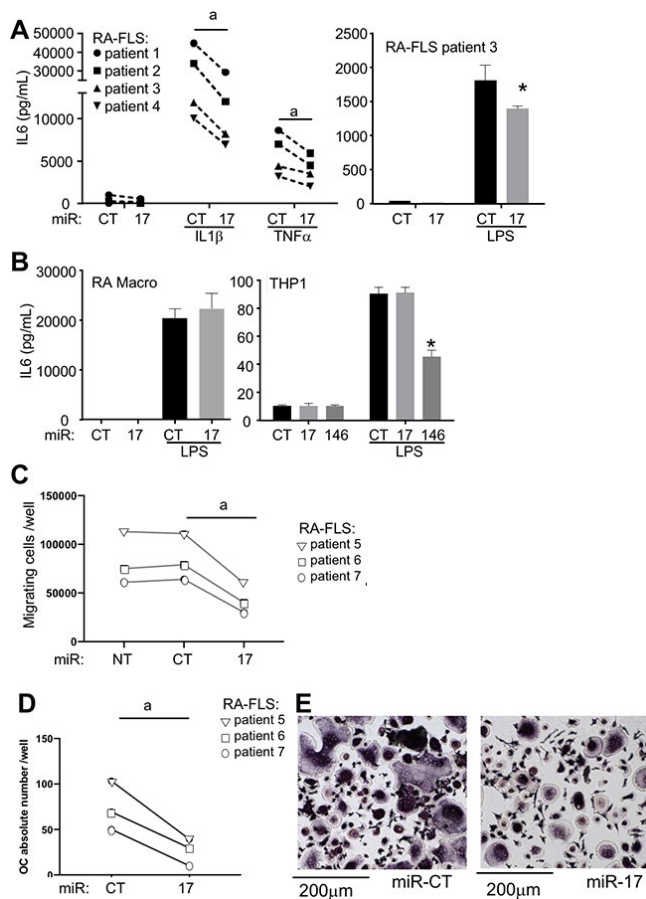


Figure 4. MicroRNA-17 suppresses interleukin-6 (IL-6) secretion in rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS). **A**, RA FLS from 4 patients were transfected with the microRNA-17-5p mimic (miR-17) or the irrelevant microRNA (miRNA) control (miR-CT) and then stimulated for 24 hours with IL-1 β , tumor necrosis factor (TNF), or lipopolysaccharide (LPS). Secretion of IL-6 in the culture supernatants is shown. **B**, RA macrophages (Macro) were generated from RA synovial fluid monocytes, transfected with the indicated miRNA, and stimulated with LPS. THP-1 monocytic cells were differentiated, transfected with the indicated miRNA, and stimulated with LPS. Secretion of IL-6 in the culture supernatants was assessed by enzyme-linked immunosorbent assay. **C**, Peripheral blood mononuclear cells were collected and counted after 4 hours of migration through a 5.0- μ m Transwell system with control- or miR-17-transfected RA FLS from 3 patients. **D**, CD14 $^{+}$ cells from healthy donors were differentiated into osteoclasts (OCs) and exposed to conditioned medium (1:4 dilution) from control- or miR-17-transfected RA FLS (stimulated for 48 hours) from 3 different patients. The number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclasts is shown. In **A** (right panel) and **B**, results are shown as the mean \pm SEM. $a = P < 0.05$ by paired Wilcoxon-Mann-Whitney test; $* = P < 0.05$ versus control by Mann-Whitney U test. **E**, TRAP staining is shown. Original magnification $\times 20$. 146 = miR-146a-5p (positive control); NT = not transfected. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.41441/abstract>.

others. JAK1 and STAT3 transcripts were significantly reduced in cells transfected with miR-17 (Figure 5A). Other JAK and STAT transcripts were not down-regulated by miR-17. Interestingly, we

confirmed the reduction in JAK1 and STAT3 expression at the protein level after miR-17 transfection. Expression of other JAK and STAT proteins was unaltered (Figure 5B). Expression of miR-17 did not alter the expression of genes implicated in the TNF signaling pathway (*TRAF2*, *TRAF6*, *NF κ B*, *ASK1*) or of Smad family members (Figure 5B and Supplementary Figure 4, <http://onlinelibrary.wiley.com/doi/10.1002/art.41441/abstract>).

In order to confirm that miR-17 directly targeted JAK1 and STAT3 mRNA, we performed 3'-untranslated region (3'-UTR) assays in HEK 293T cells. The results confirmed that miR-17 was able to significantly reduce JAK1 and STAT3 3'-UTR luciferase activity (Figure 5C).

The above findings suggested that miR-17 directly targets the JAK1/STAT3 pathway rather than the TNF signaling pathway. To confirm this hypothesis, we next used promoter-luciferase reporter plasmids driven by NF- κ B or STAT3. Interestingly, we observed a significant decrease in STAT3 activity after miR-17 transfection, but no effect on NF- κ B activity was seen (Figure 5D).

TNF activates an autocrine loop involving IL-6 family members.

To further elucidate the miR-17 mechanism of action, we compared the effects of several JAK/STAT signaling pathway inhibitors to the effects of miR-17. First, TNF increased STAT3 activation/phosphorylation (Tyr⁷⁰⁵/phospho-STAT3), and the JAK1 inhibitor ruxolitinib totally abolished STAT3 activation in RA FLS (Figure 5E). As observed with miR-17, the JAK1 inhibitor also reduced IL-6 secretion induced by TNF (Figure 5F). Similar effects were observed with neutralizing antibodies against gp130, the common receptor chain used by all IL-6 family cytokines, or against the cytokine LIF. In contrast, IL-6R or OSM neutralization did not alter IL-6 secretion (Figure 5F), in accordance with their absence in RA FLS (Figure 5A). Therefore, TNF appeared to induce IL-6 secretion partly through activation of an autocrine-amplifying loop involving LIF, gp130, JAK1, and STAT3. When ruxolitinib and miR-17 lipoplex were combined, there was no additional antiinflammatory effect, compared to the effect of a single treatment (Figure 5G). These results suggest that both share the same targets, JAK1 and STAT3.

Suppression of the JAK1/STAT3 pathway by miR-17 in ankles of mice with CIA.

Finally, we examined whether miR-17 lipoplex exerted its effect on arthritic paws through the same mechanism as in RA FLS (via JAK1 and STAT3 targeting). We assessed expression levels at an early time point (day 4) and observed a significant reduction in JAK1 and STAT3 transcripts (Figure 6A). Tyk2 expression was also significantly reduced, but STAT4 was not (Figure 6A). In addition, we confirmed the reduction of STAT3 activation by performing phospho-STAT3 immunohistochemistry in ankle tissue (Figure 6B).

DISCUSSION

In this report, we have presented evidence of an antiinflammatory role of miR-17. Among a panel of identified miRNAs

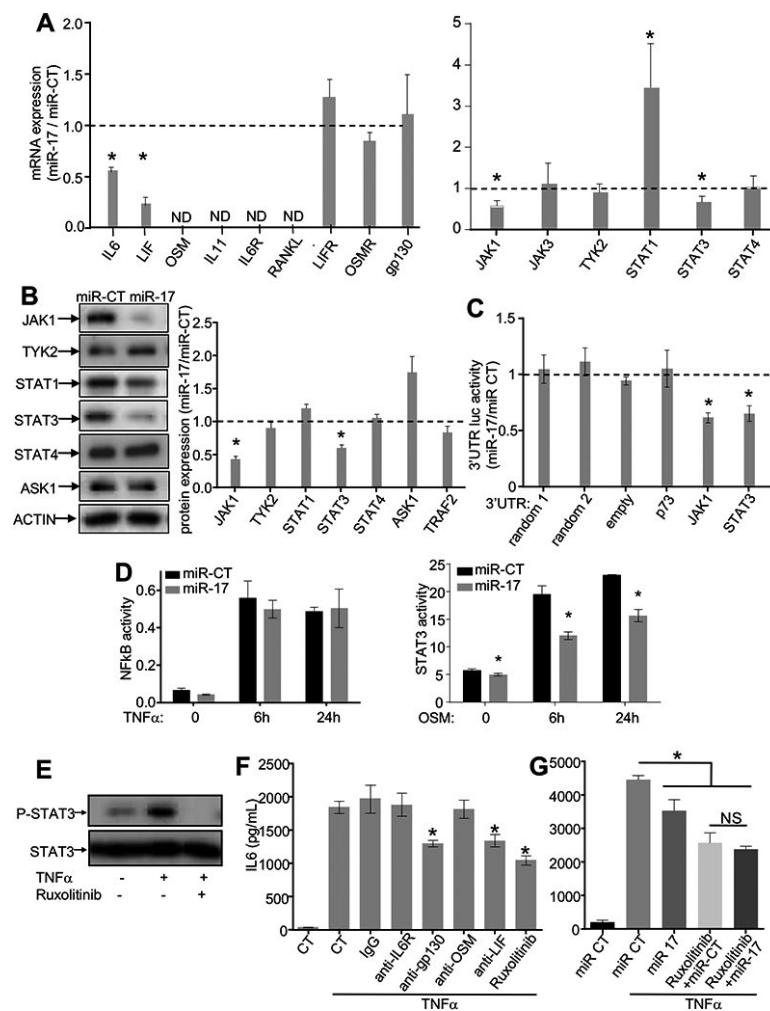


Figure 5. MicroRNA-17 targets JAK1 and STAT3. **A**, RA FLS from 4 patients were transfected with miR-CT or miR-17, stimulated with TNF, and analyzed for expression of the indicated genes by reverse transcription–quantitative polymerase chain reaction. **B**, RA FLS from 3 patients were transfected with miR-CT or miR-17, stimulated with TNF, and analyzed for expression of the indicated proteins by Western blotting. Actin was used as an internal control. Representative images from 1 patient (left) and quantification of the protein expression levels from the 3 patients (right) are shown. **C**, HEK 293T cells were transfected with luciferase reporter plasmids containing the indicated 3'-untranslated region (3'-UTR) together with miR-17 or miR-CT. Luciferase activity was measured 24 hours after transfection. **D**, HEK 293T cells were transfected with promoter-luciferase reporter plasmids driven by NF-κB or STAT3, together with miR-17 or miR-CT, and stimulated with TNF or oncostatin M (OSM) for the indicated amount of time. **E**, RA FLS were stimulated for 24 hours with TNF and the JAK1 inhibitor ruxolitinib, as indicated. Phosphorylation of STAT3 (Tyr⁷⁰⁵) was analyzed by Western blotting. **F**, RA FLS were stimulated for 24 hours with TNF-neutralizing antibodies and ruxolitinib, as indicated. Secretion of IL-6 in the culture supernatants was assessed by enzyme-linked immunosorbent assay (ELISA). **G**, RA FLS were transfected with miR-17 or miR-CT and then stimulated for 24 hours with TNF and ruxolitinib, as indicated. Secretion of IL-6 in the culture supernatants was assessed by ELISA. In **C–G**, One representative experiment of ≥ 3 independent experiments is shown. Bars show the mean \pm SEM. * $P < 0.05$ by Mann-Whitney U test. ND = not detected; LIFR = leukemia inhibitory factor receptor; CT = control without antibody; IgG = control with irrelevant antibody (see Figure 4 for other definitions).

associated with the erosive phenotype of RA, we chose to study, in depth, the effects of miR-17 and its mechanism of action. It has previously been shown that miR-17 exerts an antiinflammatory effect in RA FLS (10). Here, we further elucidated the mechanisms involved in mediating the antiinflammatory effects of miR-17 both in RA FLS and in the CIA murine model. The transfection of miR-17 into mouse ankles significantly reduced secretion of proinflammatory cytokines (IL-6, IL-1 β) as well as inflammatory cell infiltration. Of interest, both innate immune cells (macrophages and osteoclasts)

and adaptive immune cells (B and T lymphocytes) known to be involved in RA pathophysiology were significantly reduced in the synovial tissue, suggesting a wide-ranging role of miR-17. Despite the variability of arthritis disease course across experiments, previously described in the CIA model (18), the antiinflammatory effect of miR-17 has consistently been demonstrated here.

The effect exerted by miR-17 in reducing IL-6 expression directly involved the JAK1/STAT3 pathway, and our data suggest that the suppression of the JAK1/STAT3 axis was the

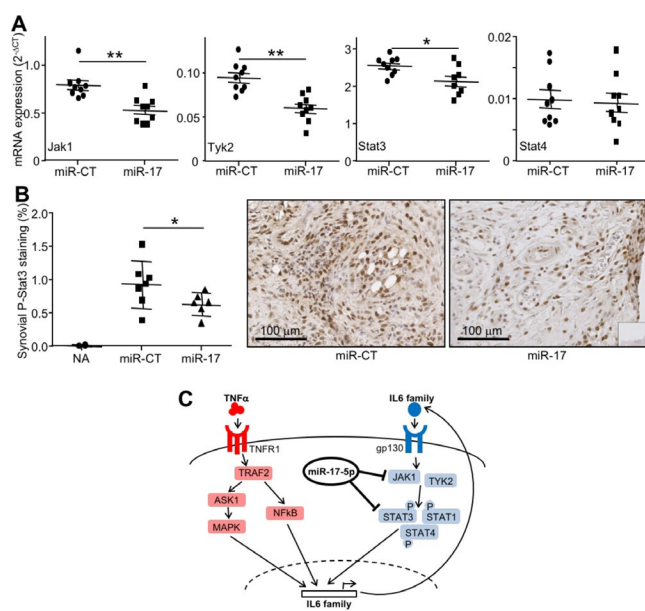


Figure 6. MicroRNA-17 lipoplex suppresses the JAK1/STAT3 pathway in the ankles of mice with collagen-induced arthritis (CIA). **A**, On day 4, injection of miR-17 into the ankles of mice with CIA ($n = 6$ or $n = 9$) significantly reduced the expression of *Jak1*, *Tyk2*, and *Stat3*, but not *Stat4*, compared to control-injected mice ($n = 7$ or $n = 9$), as assessed by reverse transcription–quantitative polymerase chain reaction. **B**, On day 7, miR-17 significantly reduced synovial activation/phosphorylation of STAT3 as assessed by immunohistochemistry. Representative images (right) and quantification of the stained areas (left) are shown. Symbols represent individual ankles; bars show the mean \pm SEM. * = $P < 0.05$; ** = $P < 0.005$, by Mann-Whitney U test. **C**, Model shows a proposed mode of action of miR-17 in arthritic joints. Overexpression of miR-17 in synovial fibroblasts does not impact TNF signaling but targets an autocrine-amplifying loop involving IL-6 family members and ≥ 2 signaling partners, JAK1 and STAT3. NA = nonarthritic, noninjected ankles ($n = 2$) (see Figure 4 for other definitions). Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.41441/abstract>.

primary mechanism of action (Figure 6C). In contrast to previous findings, we did not observe any direct effect of miR-17 on the TNF pathway. Indeed, miR-17 action did not involve TNF or NF- κ B, as demonstrated by promoter-luciferase assays. Moreover, one of the identified targets, ASK1, was not affected by miR-17 transfection in either in vitro or in vivo experiments. In contrast to results obtained in mesenchymal stem cells (19), we did not observe down-regulation of the Smad pathway by miR-17 in RA FLS.

Transfection with miR-17 also protected the mice from bone erosions and cartilage damage. This protective effect was presumably the result of reduced inflammation and immune cell infiltration, reduction in osteoclasts, and expression of RANKL in the synovial tissue. This might be due to the silencing of STAT3, as it is suggested that STAT3 is directly responsible for RANKL expression (4,20,21). However, RANKL expression was not detectable in RA FLS, as previously shown (22). These findings possibly

implicate other prominent cell sources of RANKL, such as lymphocytes and monocytes (23,24).

In RA, TNF and IL-6 are known to be prominent proinflammatory cytokines involved in its pathophysiology and expressed especially in the synovial tissue, along with IL-1 and IL-17 (25). RA FLS are known to be an important source of IL-6 production within the inflamed synovium (26). Of note, when we stimulated RA FLS with TNF, we observed a significant reduction not only in IL-6 secretion, but also in the other IL-6 family cytokine LIF, by miR-17. We compared the effect of miR-17 to that of the JAK1 inhibitor ruxolitinib (27) and observed a similar effect, suggesting a common mechanism of action. TNF is well known to induce expression of IL-6 through activation of NF- κ B and MAPKs (28). However, recent studies suggest that IL-6 production and secretion also involve an autocrine feedback loop through other IL-6 family members, especially LIF, LIFR, and different signaling partners such as JAK1, Tyk2, STAT3, and/or STAT4, driving a sustained activation and secretion of proinflammatory mediators in RA FLS (3). RA FLS do not express IL-6R, although they express LIFR composed of gp130 and LIFR chains (3,4,29). We believe that miR-17, by directly targeting at least the JAK1/STAT3 axis, indirectly reduces LIF and IL-6 production and acts as a negative regulator of this feedback loop (Figure 6C). In our experiments, miR-17 did not affect STAT4 expression levels in either RA FLS or synovial tissue from mice with CIA. In mouse synovial tissue, Tyk2 was significantly reduced by miR-17, but this was not the case in RA FLS.

Interestingly, blocking the pathogenic effect of proinflammatory cytokines such as IL-6 (IL-6R blockade, JAK Inhibitors) (30,31) or TNF is the common strategy for RA therapy. In the present study, we confirmed that miR-17 expression was significantly decreased, especially in erosive RA phenotypes. By restoring miR-17 levels in the synovial tissue by local injection and transfection, we were able to significantly reduce inflammation and structural damage. These findings suggest that miR-17 acts as a regulator of synovial inflammation. The disruption of miR-17 expression results in a loss of inhibition of proinflammatory pathways, especially IL-6, and leads to an erosive phenotype in RA.

This study is the first to demonstrate an antiinflammatory and structural role of miR-17 in vivo. Moreover, we have described in detail the mechanisms of action of miR-17 and have demonstrated an effect of miR-17 on the JAK/STAT pathway through direct targeting of JAK1 and STAT3. Very few studies have examined the expression of miRNA locally in RA synovium, and there are currently no data on synovial miRNA expression in relation to structural damage. This work allowed us to identify a miRNA significantly associated with an erosive phenotype in early and established RA, and further applications could include the study of miR-17 as a potential biomarker of erosive RA in a dedicated cohort. Moreover, miR-17 could be envisaged as a potential local or systemic therapy in erosive RA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Najm had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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